# Corrugatin, a Lipopeptide Siderophore from *Pseudomonas corrugata**

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From the culture medium of the phytopathogenic bacterium *Pseudomonas corrugata*, a lipopeptide siderophore was isolated that comprises interesting structural elements such as cyclic condensation products of the two amino groups of 2,4-diamino butyric acid with the carboxyl group of a second amino acid, and the rarely encountered L-threo-β-hydroxy histidine.

## Introduction

Lipopeptides are secondary metabolites of many microorganisms. They consist of an oligopeptide bound to a saturated fatty acid. The peptide chain usually contains several non-proteinogenic amino acids. Macrocyclic structures formed by lactone or lactame units seem to be more common than acyclic representatives. The fatty acid can be straight or branched and often carries a R-3-hydroxyl group. Due to their amphiphilic character (hydrophobic fatty acid and hydrophilic peptide part), the lipopeptides are detergents that offer several advantages to the producing bacteria: It enables them to grow on hydrophobic surfaces (Matsuyama *et al.*, 1992) and to use hydrocarbons as carbon source by forming emulsions (Yakimov *et al.*, 1995). A formation of ion channels increases the permeability of the cell membranes, which results in the loss of $K^+$ (Hutchinson *et al.*, 1995); the fungicide activity based on this mechanism could eventually be used in human medicine (Potera, 1994). A plant deleterious effect can be reached by the destruction of the protecting wax film of leaves, which allows bacterial growth inside the plant and results in its death (Laycock *et al.*, 1991).

Bacteria frequently live in surroundings where the amount of soluble iron compounds is limited. In order to secure a sufficient supply, most of them produce $Fe^{3+}$ complexing substances, so-called siderophores. Since $Fe^{3+}$ forms octahedral complexes, three bidentate ligands have to be provided that frequently are connected by aliphatic spacers, which brings them into the correct position. A large variety of structural types has been identified ranging from simple compounds such as salicylic acid (Meyer *et al.*, 1992) to highly complex chromopeptides such as the pyoverdins of the fluorescent pseudomonads (Budzikiewicz, 1997). Yet so far, only one example is known where lipopeptides act as siderophores, viz. the ornibactins from *Pseudomonas cepacia* (Stephan *et al.*, 1993). We wish now to report a second example, **corrugatin** (**1**) from *Pseudomonas corrugata*. Corrugatin has several structural pecularities, the most notable being the rarely encountered amino acid β-hydroxy-His.

*Abbreviations.* Common amino acids, 3-letter code; OHAsp, β-threo-hydroxy Asp; Dab, 2,4-diamino butyric acid; OHHis, β-threo-hydroxy His; CAS Test: Chromazurol S-Test for $Fe^{3+}$-complexing substances (Schwyn and Neilands, 1987); NMR-techniques: COSY, correlation spectroscopy; DEPT, distortionless enhancement by polarisation transfer; HOHAHA, homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser effect; ROESY, rotating frame Overhauser and exchange spectroscopy; TOCSY, total correlated spectroscopy; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; DSS, 2,2-dimethyl-2-silapentan-5-sulfonate; TMS: tetramethylsilane; EDTA, ethylenediamine tetraacetate; MS: mass spectrometry; FAB: fast atom bombardment; GC, gas chromatography; RP-HPLC, reversed phase high performance liquid chromatography; TAP derivatives, N/O-trifluoroacetyl amino acid isopropyl esters.

* Part LXXVII of the series „Bacterial constituents". For part LXXVI see Budzikiewicz *et al.* (1998).

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*Pseudomonas corrugata* is a soil-borne phytopathogenic bacterium first isolated in 1978 from infected tomato (Scarlett *et al.*, 1978) and later also isolated from pepper plants (Lopez *et al.*, 1994). In contrast, by two Australian strains the take-all disease of wheat was diminished significantly and the growth of diseased plants was increased (Ryder and Rovira, 1993); *P. corrugata* may be useful for biocontrol applications (Kovacevich and Ryder, 1991). *P. corrugata* cells cause hypersensitive reactions (HR) in nonhost tobacco (Devlin and Gustine, 1992; Gustine *et al.*, 1994), and elicit phytoalexin (medicarpin) biosynthesis (Gustine *et al.*, 1990) and active oxygen burst in white clover (Devlin and Gustine, 1992). An uncharacterized phytoalexin elicitor (Gustine *et al.*, 1990) and two HR elicitors from *P. corrugata* were reported (Gustine *et al.*, 1994; Gustine *et al.*, 1995). The taxonomical placement of *P. corrugata* within the genus *Pseudomonas* is controversial; by some authors it was included in the rRNA homology group I related to the fluorescent pseudomonads (for a detailled discussion see: Sutra *et al.*, 1997, who also give an emended description of *P. corrugata*). The siderophore pattern, *viz.* absence of pyoverdins and formation of a lipopeptide siderophore should suggest a closer relationship with *P. cepacia* (rRNA homology group II).

## Material and Methods

### *Bacterial strain*

*Pseudomonas corrugata* used in this work was isolated from alfalfa roots (Lukezic, 1979).

### *Chemicals*

$H_2O$ was deionized, distilled twice and filtered through XAD-4 resin (Serva, Heidelberg) and through a sterile filter (4 μm). Solvents were distilled once. Chemicals had p. a. quality. $(^{15}NH_4)_2SO_4$ (Campro, Emmerich) contained 99.9% $^{15}N$.

### *Separation techniques*

Adsorption resins: XAD-2 (0.3–1.0 mm) (Serva, Heidelberg), purified by shaking with 1% methanolic HCl, washing with $H_2O$ until neutral and activated by sucking air through the packed column for 6 hrs, interrupted every half hr by moistening with $H_2O$; Sep-Pak $RP_{18}$ cartridges (Waters, Milford, MA, USA). Low pressure chromatography: Sephadex G-15 (equilibrated with $H_2O$) and peristaltic pump P-3 (Pharmacia, Uppsala, S). HPLC: Nucleosil-100 $C_{18}$ (5 μm), Polygosil 60 $C_{18}$ (7 μm), Kromasil 100-$C_4$ (5 μm), Nucleodex-β-OH (Macherey & Nagel, Düren), HPLC pump 64 (Knauer, Berlin), buffer solutions (1) 0.58 $H_4EDTA$ + 0.4 ml 25% $NH_3$/l (pH 6.0), (2) 3.9 ml triethylamine + 3.9 ml formic acid (99.8–100%)/l (pH 3.1), (3) 2.5 ml triethylamine + 6 ml glacial acetic acid/l (pH 3.9). GC: HRGC 4160 with FID detector, He (Carlo Erba, Milano, I) with a Chirasil-L-Val column (Chrompack, Frankfurt). High voltage paper electrophoresis: HVE-60600 with paper MN 261 (Camag, Muttenz, CH), 2 kV, references glucose and desferal, buffers 25 mM phospate (pH 6.9), 0.1 M citrate/HCl (pH 4.0), 0.1 M acetate (pH 2.7) (Poppe *et al.*, 1987).

### *Spectroscopy*

UV/Vis: Lambda 7. IR: Spektrophotometer 283 (both Perkin-Elmer, Überlingen). GC-MS: Incos 50 XL with GC Varian 3400, capillary column CB SE-54 (Finnigan-MAT, Bremen). FAB-MS: HSQ-30 (Finnigan-MAT, Bremen) with FAB-gun (Ion-Tech Ltd., Teddington, GB), FAB-gas Xe, collision gas Ar. NMR: AM 300 and DRX 500 (Bruker, Karlsruhe), chemical shifts relative to HDO (4.8 ppm at 25 °C and 5.0 ppm at 5 °C) for $^1H$, and relative to DSS using the relation δ(TMS) = δ(DSS) −1.61 ppm for $^{13}C$.

### *Bacterial cultures*

Culture medium: 13 g Na D-gluconate in 500 ml $H_2O$, 2.5 g $KH_2PO_4$ + 1.5 g $K_2HPO_4.2H_2O$ in 150 ml $H_2O$ (pH 7.0), 5 g $(NH_4)_2SO_4$ + 0.5 g $MgSO_4$ in 150 ml $H_2O$ and 1 g $Na_4EDTA$ in 200 ml $H_2O$. The solutions were sterilized separately (130 °C, 20 min, 200 kPa), and except for the EDTA-solution (see below) poured together while still hot. 200 ml culture medium without EDTA were inoculated with *Pseudomonas corrugata* from an agar culture, grown for 24 hrs and kept in the refrigerator at 5 °C. For the production of **1,** 20 Erlenmeyer flasks (500 ml) with indentations were filled with 200 ml culture medium (without EDTA) each and inoculated with 1 ml each from the stored culture. The flasks were

shaken (100 rpm) at 25 °C. After 24 hrs, 50 ml of the sterile EDTA solution were added. After further shaking for 40 hrs, the cells were removed by tangential filtration, and 6 M HCl (to bring the pH to 6.0–6.5) and 0.2 g $NaN_3$/l (to stop further bacterial growth) were added to the combined solutions. For the isolation of ferri-**1**, 10 ml 5% $Fe^{3+}$-citrate solution/l culture medium was added before the tangential filtration (subsequent treatment as described above). For the production of $^{15}N$-labelled **1**, only 2 g (instead of 5 g) $(^{15}NH_4)_2SO_4$/l were added to the culture medium.

*Isolation of* **1**

The culture solution was adsorbed on activated XAD-2 resin (column bed 5x60 cm) and subsequently washed with 5 l $H_2O$. For desorption the resin was shaken in the column with portions of 300 ml $H_2O/CH_3OH$ 1:1 (v/v) until the solvent was not colored anymore. The yellow solution was then concentrated to ca. 25 ml (rotatory evaporator, 35 °C), brought onto a Sephadex column (filled 3x65 cm) and eluted with $H_2O$ (flow rate 1.2 ml/min); detection at 254 and 435 nm. Several fractions were obtained, but only the first one was CAS-positive. This fraction was adsorbed on a Sep-Pak cartridge and **1** was desorbed with $H_2O$/isopropanol 4:1 (v/v). In RP-HPLC (Nucleosil, solvent $CH_3OH$/buffer 1, gradient 3 to 78% v/v $CH_3OH$) only one peak was observed. Yield 10–12 mg **1** from 1 l culture medium.

*Amino acid analysis*

Hydrolysis (6 N HCl, 110 °C), TAP derivatization GC-MS and GC analysis on a chiral column was performed as described earlier (Jacques *et al.*, 1995).

Dansylation of the 7-day hydrolysate (1 mg **1**): After removal of HCl by distillation i.v., the residue was dissolved in 12 ml 40 mM $Li_2CO_3$ buffer (pH 9.5) and a solution of 6 mg dansyl chloride in 9 ml acetonitrile was added. The mixture was kept over night in the dark at room temp., treated with 0.2% ethylamine in $H_2O$ and brought to dryness, redissolved in 20 ml 0.1 M $CH_3COOH$, adsorbed on a Sep-Pak cartridge, and washed with 0.1 M $CH_3COOH$. The dansylated amino acids were then desorbed with isopropanol $H_2O$ 1:1 (v/v) and analyzed by RP-HPLC on Polygosil, using a gradient of isopropanol/buffer 3 going from 10 to 50% v/v isopropanol. For the identification of OHHis (which could not be detected after TAP derivatization (cf. Sharman *et al.*, 1995), **1** was hydrolyzed for 70 hrs; 3 mg of the dry hydrolysate were treated with 25 ml $Li_2CO_3$ buffer and 25 ml dansyl chloride solution (see above) for 1 hr at 37 °C. The reaction was quenched with 5 ml 10% HCOOH, acetonitril was distilled off, the residue adsorbed on Sep-Pak, washed with diluted HCOOH and $H_2O$, desorbed with 2 ml isopropanol/$H_2O$ 2:1 (v/v), and brought to dryness. Dansylated OHHis was isolated by chromatography on Kromasil using a gradient $CH_3OH$/20 mM $CH_3COOH$ going from 20 to 100% $CH_3OH$ v/v (detection at 254 nm) and then rechromatographed under the same conditions. Comparison with authentic material (Weber, 1997) and coinjection identified the isolated product as dansyl-threo-OHHis. Subsequent chromatography on Nucleodex using a gradient $CH_3OH$/50 mM $CH_3COONH_4$ buffer (pH 6.1) going from 10 to 100% $CH_3OH$ v/v (detection at 254 nm) and comparison with authentic material including coinjection established the L-configuration.

Free amino groups were detected by treating 1 mg **1** dissolved in 2 ml 40 mM $Li_2CO_3$ buffer with 1.5 mg dansyl chloride in 1 ml acetonitril for 2 hrs at 37 °C. After addition of 1 ml 2% ethylamine in $H_2O$ and after 5 min of 0.5 ml 10% HCOOH, acetonitril was distilled off, the residue was adsorbed on a Sep-Pak cartridge, washed with $H_2O$, and eluted with isopropanol/$H_2O$ 1:1 (v/v). After removal of isopropanol by distillation, the dansylated **1** was hydrolyzed (cf. above) for 21 hrs. After removal of HCl and concentration, the residue was adsorbed on Sep-Pak, non-dansylated amino acids were desorbed with $H_2O$ and the dansylated ones with isopropanol/$H_2O$ 1:1 (v/v). RP-HPLC analysis was conducted on Nucleosil with a gradient isopropanol/buffer 2 going from 10 to 35% isopropanol (column temp. 50 °C).

For hydrazinolysis, 0.5 g **1** and 0.5 ml water-free $N_2H_4$ were heated to 89 °C for 18 hrs. Excess $N_2H_4$ was removed i.v., and the residue was dissolved in 1 ml $H_2O$ and extracted for 1 hr with 1 ml freshly distilled benzaldehyde. The phases were separated by centrifugation, the aqueous phase was extracted twice with 1 ml ether each, brought to dryness i.v. and TAP derivatized (see above).

For the location of D/L-Ser, 20 mg of **1** were hydrolyzed at 90 °C with 6 N HCl for 15 min. After bringing to dryness i.v., chromatography of the residue on a Biogel P2 column with 0.1 N $CH_3COOH$ yielded 4 fractions that were analyzed by FAB-mass spectrometry. The 3rd fraction contained i.a. a fragment with only one Ser, *viz.* the N-terminal portion octanoic acid-OHHis-Dab-Dab-Ser. TAP-derivatization after total hydrolysis (*v. supra*) and subsequent chromatography on a Chirasil-L-Val column indicated that the N-terminal Ser had the L-configuration. This result was confirmed indirectly by cleavage of **1** with astacin (Krauhs *et al.*, 1982): Astacin cleaves peptides on the amino side of small uncharged residues as *i.a.* Ser. 1 mg of **1** dissolved in 1 ml of a 0.1 M solution of $NH_4HCO_3$ (pH 8.0) was incubated with 0.02 mg astacin for 6 hrs at 25 °C. After work-up as above, a peptide with the mass (as determined by FAB-MS) 480 was isolated. This indicates that **1** was cleaved only before the first Ser starting from the N-terminus. Since proteases from representatives higher up in the animal kingdom generally recognize only proteinogenic L-amino acids (otherwise two cleavage products would be expected), it can be assumed that the Ser closer to the N-terminus is L-configurated.

## Results and Discussion

### *Bacterial growth and isolation of corrugatin*

*Pseudomonas corrugata* can be grown on an artificial mineral medium with gluconate as carbon source. Of essential importance is the control of the concentration of Fe salts in the medium. Siderophore production starts only below a species-dependent level, but too low amounts reduce or even stop the cell growth. Several strategies have been developed to guarantee an adequate Fe level; a practical approach applied successfully here was to use commercial Na gluconate which contains a sufficient amount of iron (13 g/l give a concentration of ca. 100 μg/l), but to add $Na_2EDTA$ as a competing iron chelating agent after 24 hrs of bacterial growth. Isolation of **1** was achieved by several consecutive chromatographic steps: **1** can be adsorbed on XAD-2 resin, freed from salts and from polar bacterial metabolites by passing $H_2O$ through the column, and finally desorbed with $CH_3OH/H_2O$. Chromatography on Sephadex yields several fractions, one of which was CAS-positive and could be purified further by adsorption on Sep-Pak.

### *Characterization of corrugatin*

The UV/Vis spectrum of **1** shows a strong absorption band at 260 nm ($\varepsilon = 5000\ l.mol^{-1}.cm^{-1}$ at pH 3.0, 4000 at pH 7.4) and weak ones ($\varepsilon \sim 800\ l.mol^{-1}.cm^{-1}$) at 375 and 450 nm. The position of the bands is pH independent. The low intensity of the bands at 375 and 450 nm suggests that they are due to charge-transfer transitions responsible for the yellow color of **1** (**1** contains no chromophore which would absorb in this region). **1** shows yellow fluorescence under UV (366 nm). In the IR spectrum, amide bands at 1652 and 1545 $cm^{-1}$, but no ester bands can be observed. By FAB-MS, the molecular mass of **1** could be determined as 998 u, that of its yellow $Fe^{3+}$ complex as 1051 ($M + Fe^{3+} - 3\ H^+$). The molecular mass of **1** grown with $(^{15}NH_4)_2SO_4$ as nitrogen source was 1011 u, which indicates that 13 N atoms are contained in **1**. High voltage paper electrophoresis (cf. Poppe *et al.*, 1987) showed that **1** has two negative charges at pH 6.9 and 4.0 and only one negative charge at pH 2.7. Since **1** has one free $NH_2$-group (see below: γ-$NH_2$ of Dab) that is protonated in the above pH-range three free COOH-groups must be present as well as a function that is protonated completely at pH 2.7 (see below: OHHis).

An amino acid analysis of the TAP derivatives after hydrolysis by GC-MS and GC with a chiral column gave the following results: After 21 hrs hydrolysis, L-β-threo-OH-Asp, L-Dab and D- and L-Ser could be identified. Prolonged hydrolysis (45 hrs) produced a higher yield of OHAsp. This is typical for the presence of a cyclic condensation product of an amino acid with Dab (cf. below). By HPLC of the dansylated hydrolysate on Polygosil with buffer 3 an additional peak could be identified as β-hydroxy-His by comparison with authentic material. β-Hydroxy-His has been encountered rarely in nature. It exists in 4 diastereomeric forms, viz. D/L-erythro and D/L-threo. The L-erythro form is present in the antibiotic bleomycin (Koyama *et al.*, 1973), and the L-threo form in exochelin MN (Sharman *et al.*, 1995) from *Mycobacterium neoaurum* and in a pyoverdin from *Pseudomonas fluorescens* (Hancock *et al.*, 1993;

Budzikiewicz *et al.*, 1997). For an unambiguous identification of the stereoisomer present in **1**, all four enantiomers were synthesized (Weber, 1997). Dansyl-β-hydroxy-His was isolated by chromatography on Kromasil with $CH_3OH/CH_3COOH$. By comparison with a mixture of synthetic erythro- and threo-β-hydroxy-His and by coinjection the configuration of the natural OHHis could be shown to be threo. Chromatography on the chiral Nucleodex column with $CH_3OH$/50 mM $CH_3COONH_4$ in comparison with the synthetic material confirmed the L-configuration. As the other bacterial examples, **1** contains L-threo-β-hydroxy-His.

By dansylation of **1** and subsequent total hydrolysis, 2-amino-4-dansylaminobutyric acid could be identified by HPLC using authentic comparison material. Hence, at least one Dab with a free γ-amino group (cf. above the electrophoresis results) is incorporated into **1**. A hydrazinolysis experiment showed that OH-Asp is the C-terminal amino acid with two free COOH-groups.

According to these results, the peptide part of **1** contains D- and L-Ser in equal amounts, Dab (at least one with a free γ-$NH_2$-group), L-threo-β-OH-His, and C-terminal L-threo-β-OH-Asp with free COOH-groups. The molecular mass of **1** and the presence of 13 N-atoms in the molecule suggest that some of these amino acids are present more than once.

### *NMR-spectroscopic studies*

The various NMR techniques (see, e.g., Evans, 1995) as applied to peptide siderophores were described earlier in some detail (Schaffner *et al.*, 1996) and will be briefly summarized here. H,H-COSY shows the $^3J$-coupling of H-C-C-H, while $^4J$-coupling within one amino acid residue (amide bonds interrupt the scalar H,H-coupling) can be detected by HOHAHA and/or TOCSY, mix time 40 ms. Direct ($^1J$) C,H-connections can be determined by a HMQC experiment. Accordingly, quaternary C show no HMQC cross peaks; they can be identified by HMBC experiments which can be optimized for $^2J$- and $^3J$-C,H-coupling. CH-, $CH_2$- etc. groups can be identified by DEPT experiments. Peptide sequencing is possible by the ROESY, mix time 140 ms, technique, which by resorting to the Nuclear Overhauser Effect allow a correlation between an NH-proton and the spacially close (d ≤ 300 pm) α- and β-protons of the preceding amino acid (-C**H**-C**H**-CO-N**H**-).

### $^1$H-measurements

Table I gives the data obtained in unbuffered $D_2O$. Using buffered (pH 4.3) $D_2O$ results in shifts ≤ 0.1 ppm with the exception of H-2-OHHis (see below). The various signals were identified by comparison with literature data and by the correlation techniques mentioned below. OHHis, the two tetrahydropyrimidine systems and octanoic acid will be discussed separately below.

Table I. 300 MHz $^1$H-NMR data of **1**.

| C-Protons[a] | δ [ppm] |
|---|---|
| H-2-OHHis | 7.90 |
| H-4-OHHis | 7.33 |
| β-OHHis | 5.36 |
| α-OHHis | 5.04 |
| α-OHAsp$^1$ | 4.91 |
| β-OHAsp$^1$ | 4.52 |
| α-OHAsp$^2$ | 4.84 |
| β-OHAsp$^2$ | 4.63 |
| α-Ser$^1$ | 4.74 |
| β-Ser$^1$ | 3.93/4.02 |
| α-Ser$^2$ | 4.60 |
| β-Ser$^2$ | 3.98 |
| α-Dab$^1$ | 4.62 |
| β-Dab$^1$ | 2.21/2.35 |
| γ-Dab$^1$ | 3.20 |
| α-Dab$^2$ | 4.52 |
| β-Dab$^2$ | 2.16/2.30 |
| γ-Dab$^2$ | 3.34/3.64 |
| α-Dab$^3$ | 4.47 |
| β-Dab$^3$ | 2.16/2.30* |
| γ-Dab$^3$ | 3.34/3.64* |
| Oct-2 | 2.36 |
| Oct-3 | 1.54 |
| Oct-4 | 1.16 |
| Oct-5/6 | 1.27 |
| Oct-7 | 1.31 |
| Oct-8 | 0.93 |
| NH-Protons[b] | |
| NH-Dab$^2$ | ≈9.5 |
| NH-Dab$^1$ | 8.92 |
| NH-Ser$^2$ | 8.84 |
| NH-Ser$^1$ | 8.82 |
| NH-OHAsp$^1$ | 8.74 |
| NH-OHHis | 8.66 |
| NH-OHAsp$^2$ | 7.87 |

[a] In $D_2O$ at 25 °C, relative to HOD, δ = 4.8 ppm.
[b] In 100 mM $KH_2PO_4$, pH 4.3 at 5 °C, relative to HOD, δ = 5.0 ppm.
* Identification not unambiguous.

For the two OHAsp units, the corresponding α- and β-H-signals could be correlated by H,H-COSY and the coupling constants (4.91 ppm – $^3J$ 4.3 Hz, 4.84 ppm – 2.2 Hz). Due to the neighboring COOH-group, the β-H show an upfield shift (0.07 ppm) when going to pH 4.3; a further shift at pH 3.0 cannot be determined because of a superposition with other signals.

The signals of one of the Ser present no pecularities: The α-triplet at 4.60 ppm corresponds to the β-doublet at 3.98 ppm. H,H-COSY connects the α-triplet at 4.74 ppm with a multiplet centered at 3.93 and 4.02 ppm. The β-protons of the other Ser are obviously not equivalent which points towards a hindered rotation. The shift of the β-$CH_2$-protones demonstrates that both hydroxyl groups are free; lactone formation would result in a downfield shift of ~0.5 ppm.

Only one Dab shows the expected signals (connected by H,H-COSY and HMBC to overcome the partial coincidence with other signals), esp. a triplet (3.2 ppm) for the γ-$CH_2NH_2$-group, in accordance with the isolation of the γ-dansylated Dab (see above). The two other Dab residues show the expected signals for the α- and β-protons, but for the γ-$CH_2$-group two broad multiplets are centered at 3.34 and 3.64 ppm. The non-equivalence of these $CH_2$-protons indicates a sterical fixation, as it had been observed for the condensation of the $NH_2$-group with the CO-group of the preceding amino acid; they will be discussed below.

Measurement of **1** in $H_2O/D_2O$ 9:1 with suppression of the $H_2O$-signal allowed the identification of all amide NH-groups by HOHAHA- and TOCSY-experiments (coupling with the α-, β- and γ-protons of the respective amino acids). Noteworthy is the position of the NH-signal of one of the OHAsp-residues (7.87 ppm, 0.8–1.0 ppm at higher field compared to the rest of the NH-signals). This was observed before for a free C-terminal amino acid (Seinsche *et al.*, 1993; Michalke *et al.*, 1996) in agreement with the result of the hydrazinolysis.

## $^{13}$C-measurements

The spectra were also measured in unbuffered $D_2O$ (Table II) and at pH 4.3. The shift differences are ≤1 ppm. OHHis, the tetrahydropyrimidine systems and octanoic acid will be discussed below. The various signals were identified by comparison with literature data, determination of the multiplicity by DEPT and correlation with the $^1$H-signals by HMBC and HMQC.

Table II. 300 MHz $^{13}$C-NMR data of **1**.

| C-Atoms[a] | δ [ppm] |
|---|---|
| NH-C=N-OHHis | 163.2 |
| α-OHHis | 57.5 |
| β-OHHis | 68.9 |
| C-2-OHHis | 137.6 |
| C-4-OHHis | 116.9 |
| C-5-OHHis | 137.9 |
| NH-C=N-OHAsp | 162.3 |
| α-OHAsp$^1$ | 56.6 |
| β-OHAsp$^1$ | 72.6 |
| β-CO-OHAsp$^1$ | 176.1 |
| CO-OHAsp$^2$ | 171.9* |
| α-OHAsp$^2$ | 59.3 |
| β-OHAsp$^2$ | 74.2 |
| β-CO-OHAsp$^2$ | 177.0 |
| CO-Ser$^1$ | 173.5 |
| α-Ser$^1$ | 56.3 |
| β-Ser$^1$ | 62.2 |
| CO-Ser$^2$ | 172.9 |
| α-Ser$^2$ | 57.0 |
| β-Ser$^2$ | 62.2 |
| CO-Dab$^1$ | 178.5* |
| α-Dab$^1$ | 52.7 |
| β-Dab$^1$ | 30.0 |
| γ-Dab$^1$ | 37.7 |
| CO-Dab$^2$ | 172.6* |
| α-Dab$^2$ | 52.7 |
| β-Dab$^2$ | 21.9* |
| γ-Dab$^2$ | 37.8 |
| CO-Dab$^3$ | 173.4* |
| α-Dab$^3$ | 53.5 |
| β-Dab$^3$ | 22.1* |
| γ-Dab$^3$ | 37.4 |
| CO-Oct | 179.5 |
| C-2 Oct | 36.3 |
| C-3 Oct | 26.2 |
| C-4 Oct | 29.2* |
| C-5 Oct | 29.0* |
| C-6 Oct | 32.1 |
| C-7 Oct | 23.1 |
| C-8 Oct | 14.6 |

[a] in $D_2O$ at 25 °C, relative to DSS, δ = –1.66 ppm.
* Identfication not unambiguous.

## threo-β-hydroxy-His

OHHis is a rare amino acid and the published NMR data ($^1$H: Haasnoot *et al.*, 1984; Hancock *et al.*, 1993; $^{13}$C: Naganawa *et al.*, 1977) do not allow an unambiguous identification. A more detailled

study was therefore necessary. The $^{1}$H-signals of the imidazole ring appear as sharp singlets. H-2 located between two N-atoms shows a pronounced pH-dependence (unbuffered 7.90, pH 4.3 8.26, pH 3.0 8.75 ppm). The α- and β-H (5.04 and 5.36 ppm) form an AB-system with $^{3}J$ = 4.0 Hz in agreement with the data published for bleomycin, while for pyoverdin Pf 244, 4.85 ppm were reported for the α-CH. The $^{1}$H/$^{13}$C-correlations as determined by HMBC and HMQC can be seen in Fig. 1. Structurally important is the observed $^{2}J$- and $^{3}J$-coupling of the α- and β-H with a $^{13}$C-signal at 163.2 ppm which indicates the presence of an amidine system (see below) rather than of an amide-CO-group.

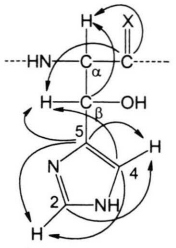



Fig. 1. Heteronuclear $^{2}J$- and $^{3}J$-coupling observed for OHHis in **1**.

## Tetrahydropyrimidine systems

The γ-$NH_2$-group of Dab in a peptide chain can undergo a cyclization reaction with the amide carbonyl of the preceding amino acid residue. This results in the formation of a tetrahydropyrimidine ring with an amidine element. The $^{13}$C-resonances of the amidine-C occur at about 10 ppm upfield as compared with amide carbonyl resonances. A condensation with the CO-group of the preceding amino acid rather than with its own CO-group (which would give a pyrroline-ring) can be confirmed by the observation of a $^{2}J$-coupling of the amidine-C and the α-CH and a $^{3}J$-coupling with the β-H of the preceding amino acid (Taraz *et al.*, 1991; Gwose and Taraz, 1992). In the $^{13}$C-spectrum of **1**, two resonances are observed whose shifts correspond to those typical for amidine systems, viz. at 163.2 and at 162.3 ppm. The signal at 163.2 ppm shows cross-signals to $^{1}$H-signals at 5.05 ($^{2}J$) and 5.36 ($^{3}J$) (α- and β-CH of OHHis), that at 162.3 to those at 4.91 ($^{2}J$) and 4.52 ($^{3}J$) (α- and β-CH of OH-Asp). In agreement with the formation of the cyclic condensation products are the $^{13}$C-signals of two Dab-β-$CH_2$-groups at ~22 ppm (Gwose and Taraz, 1992), while that of the third Dab observed at 30.0 ppm is typical for peptides containing Dab with a free γ-$NH_2$-group (Ballio *et al.*, 1994).

## Octanoic acid

The signals of the α-$CH_2$-group occurs at 2.36 ppm in agreement with other lipopeptides (e.g., Ballio *et al.*, 1994). Starting from this signal, those of the remaining chain can be identified by H,H-COSY. The position of the β-signal (1.54 ppm) indicates that a β-$CH_2$- and not a β-CHOH-group (frequently observed in lipopeptides; 3.9 ppm) is present in **1** and that the fatty acid residue is not branched. This is confirmed by the direct C,H-correlation with HMQC and the determination of the multiplicity (only $CH_2$-groups in-chain and a terminal $CH_3$) by DEPT. HMBC shows a cross peak between the α-$CH_2$-signal and that of the CO at 179.5 ppm, identifying it as that of the octanoic acid carbonyl group.

## *Sequence determination of the amino acids of* **1**

A determination of the amino acid sequence of **1** was possible since all amide protons could be identified by ROESY (see above). Starting from the C-terminal OHAsp$^{2}$ cross signals to the α-, β- and γ-H of one of the tetrahydropyrimidine rings can be observed. Going on from the amidine-NH-signal, the sequence can be extended via OHAsp$^{1}$ to Ser$^{1}$ (see Fig. 2). Since all cross signals of the two Ser are too close to each other, a connection between Ser$^{1}$ and Ser$^{2}$ is not possible. However, NH-Ser$^{2}$ shows unequivocal cross signals to the β- and γ-$CH_2$-groups of Dab$^{1}$ with the free $NH_2$-group (the cross signal to the α-CH coincides with Ser signals). A third partial sequence follows from a cross signal between the OHHis-NH and the α-$CH_2$-group of octanoic acid. Since OHHis forms part of the second tetrahydropyrimidine ring the N-terminal sequence is Oct-OHHis-Dab$^{3}$. Thus, both the N- and the C-terminal parts have been identified and hence Dab$^{1}$ -Ser$^{2}$ must form the central portion. The amino acid sequence as depicted in Fig. 2 is confirmed by several HMBC cross signals connecting a CO- signal with the α-H of the next amino acid (broken arrows in Fig. 2).

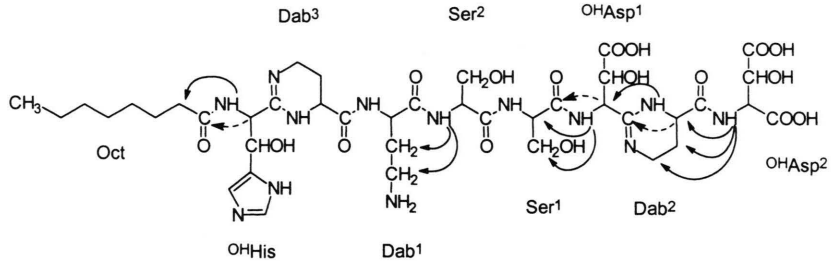



Fig. 2. Sequence-specific NOE cross signals (full arrows) and HMBC-cross peaks (broken arrows) of **1**.

*Mass spectrometric studies*

The interpretation of the mass spectral data was facilitated by comparison of the spectra of **1** and per-$^{15}$N-**1** as the mass differences indicated the number of N-atoms present in fragment ions. In the FAB-spectra several ions could be identified which can be associated with cleavages between specific amino acids units with charge retention at the N-terminal part (after Ser$^2$, *m/z* 521; Ser$^1$, *m/z* 608; OHAsp$^1$, *m/z* 739; OHAsp$^2$, *m/z* 952). Of structural interest is the loss of β-hydroxy-amino acid side chains by a McLafferty mechanism when they are located next to a tetrahydropyrimidine ring (Fig. 3). Starting from $[M+H]^+$ loss of imidazol aldehyde (from OHHis, 96 u) leads to *m/z* 902, that of oxalaldehyde (from OHAsp, 84 u) to *m/z* 924, and that of both aldehydes to *m/z* 828. The intensities which can be enhanced by collision activation, are comparable with (or even bigger than) those of the chain cleavages mentioned before. This fragmentation shows that the C-1-COOH-group of OHAsp$^1$ is incorporated into the tetrahydropyrimidine ring. Incorporation of the C-4-COOH-group would have put the β-OH group in a wrong position for the six-membered transition state necessary for the elimination process.

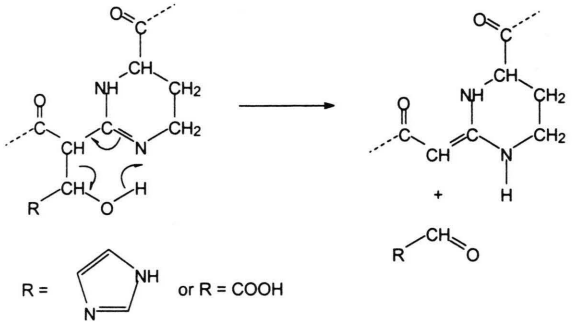
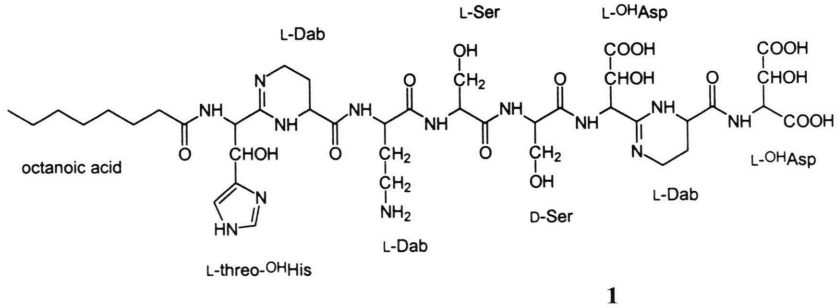



Fig. 3. Fragmentation by a McLafferty mechanism of the structural elements β-hydroxy amino acid – tetrahydropyrimidine cycle.

Collision activation of the fragment ions formed by McLafferty rearrangement yielded addional sequence specific ions with charge retention at the N-terminus (after the first tetrahydropyrimidine ring, *m/z* 238; after Dab$^1$-NH (+ 2H), *m/z* 283; after Dab$^1$-CO, *m/z* 366; after Ser$^2$-CH, *m/z* 425; after OHAsp$^2$-NH (+ 2H), *m/z* 697; numbering of the amino acids as in Fig. 2).

*Conclusions*

The sequence of the peptide chain follows from the NMR- and MS-studies. All functional groups




**1**

of the amino acids have been accounted for, viz. the β-OH-groups by the shifts of the corresponding CH-protons and the absence of an ester band in the IR-spectrum (no esters or lactones), the free $NH_2$-group of one Dab (dansylation), the free C-4-COOH group of $OHAsp^1$ (MS) and of both COOH-groups of $OHAsp^2$ ($N_2H_4$-degradation). Corrugatin has, therefore, structure **1**. Only Ser occurs both in its D- and L-form. Partial hydrolysis studies indicate that the Ser closer to the N-terminus ($Ser^2$ in Fig. 2) is L-Ser.

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